Regulation of cytoplasmic RNA stability: lessons from drosophila

Article (Accepted Version)

Article Title: Regulation of Cytoplasmic RNA Stability: Lessons from *Drosophila*

Article Type: ADVANCED REVIEW

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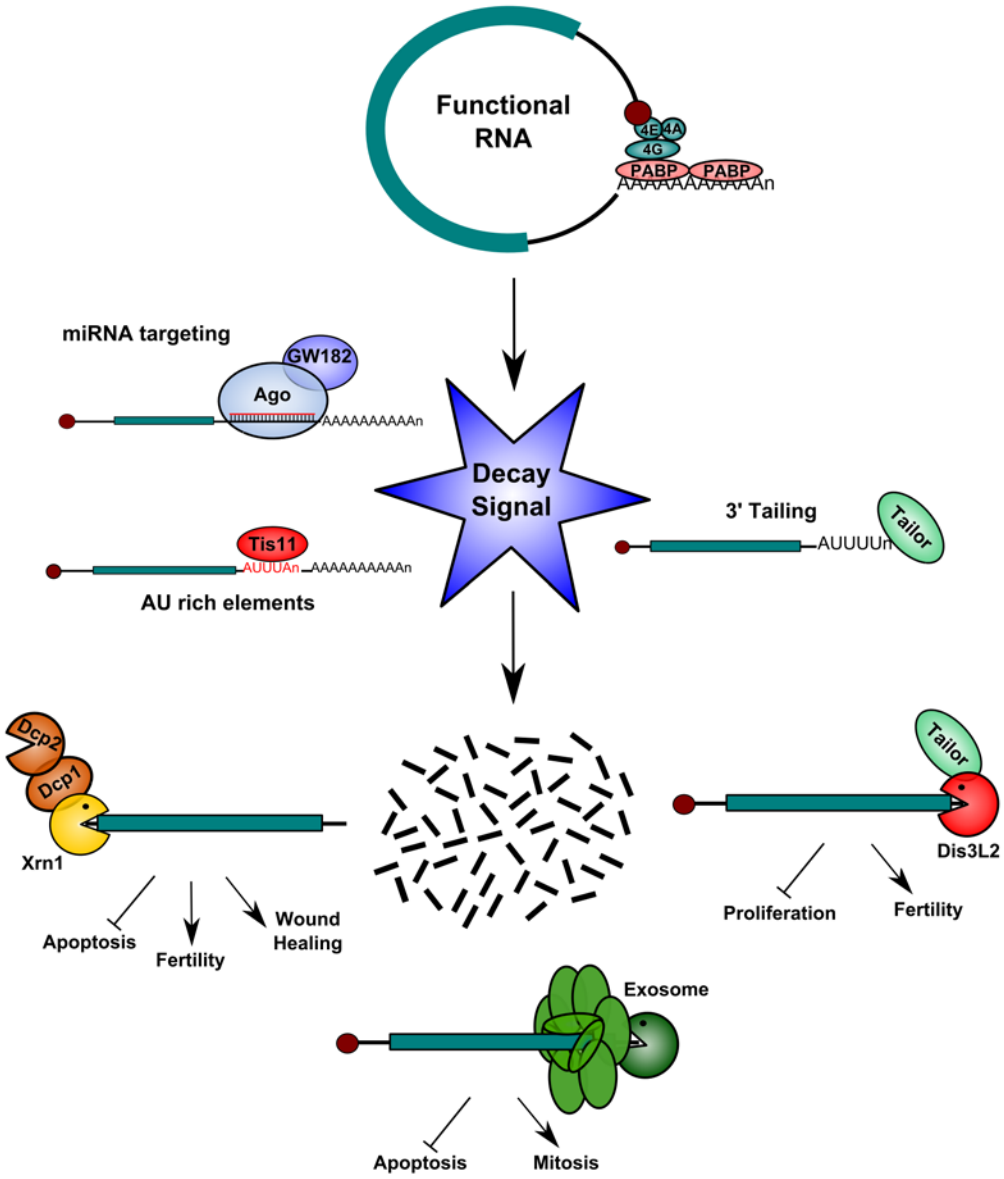
The authors declare that there is no conflict of interest.

Abstract

The process of RNA degradation is a critical level of regulation contributing to the control of gene expression. In the last two decades a number of studies have shown the specific and targeted nature of RNA decay and its importance in maintaining homeostasis. The key players within the pathways of RNA decay are well conserved with their mutation or disruption resulting in distinct phenotypes as well as human disease. Model organisms including *Drosophila melanogaster* have played a substantial role in elucidating the mechanisms conferring control over RNA stability. A particular advantage of this model organism is that the functions of ribonucleases can be assessed in the context of natural cells within tissues in addition to individual immortalised cells in culture. *Drosophila* RNA stability research has demonstrated how the cytoplasmic decay machines, such as the exosome, Dis3L2 and Xrn1, are responsible for regulating specific processes including apoptosis, proliferation, wound healing and fertility. The work discussed here has begun to identify specific mRNA transcripts that appear sensitive to specific decay pathways representing mechanisms through which the ribonucleases control mRNA stability. *Drosophila* research has also contributed to our knowledge of how specific RNAs are targeted to the ribonucleases including AU rich elements, miRNA targeting and 3' tailing. Increased understanding of these mechanisms is critical to elucidating the control elicited by the cytoplasmic ribonucleases which is relevant to human disease.

Graphical/Visual Abstract and Caption



Caption

RNA degradation in *Drosophila* is regulated by a variety of RNA-binding proteins which direct transcripts to the progressive degradation enzymes. The sensitivity of particular transcripts to degradation affects critical cellular processes such as proliferation and apoptosis.

INTRODUCTION

Throughout the genetic journey from DNA to functional protein there are a plethora of regulatory events that occur at every level to ensure that the cell, and ultimately the organism, functions correctly and maintains viability. Many of these regulatory events occur to control the levels of specific RNAs. RNA levels within the cell are maintained through delicate balancing of transcription

and degradation by ribonucleases. Previous reports have suggested that 40-50% of changes in gene expression occur at the RNA level, specifically through the control over RNA stability (Cheadle et al., 2005). Control over the amount of specific RNAs within the cell has been shown to be critical for most fundamental cellular processes, such as cell proliferation and apoptosis together with cell differentiation. This is further observed in the context of multicellular organisms, where the dysregulation of degradation of specific RNAs has been shown to result in phenotypic defects or disease (Carballo & Blackshear, 2001).

Recent work in the field of RNA stability has unveiled a number of intricate regulatory mechanisms through which the cell is able to control the amounts of specific RNAs (Perez-Ortin, Alepuz, Chavez, & Choder, 2013; Schoenberg & Maquat, 2012). These include both *cis* and *trans* acting mechanisms such as AU rich elements (AREs) within the 3' UTR and RNA binding proteins which are recruited to specific elements (such as AREs) and are able to either protect from, or promote RNA degradation. A large body of our understanding of these mechanisms has come from the use of model organisms, such as the unicellular yeast *Saccharomyces cerevisiae*, and multicellular models including mice, and the fruit fly, *Drosophila melanogaster.* Multicellular model are vital to aid our understanding of complex cellular processes as they provide a critical context missing in most cell culture work. In this review, we will discuss the current knowledge surrounding the mechanisms through which RNA stability is controlled focussing on the advances facilitated by the use of the humble fruit fly.

PATHWAYS OF RNA DEGRADATION

When the cell no longer requires a specific RNA it is signalled for degradation through a number of highly regulated steps. A messenger RNA (mRNA) is usually protected at the 5' and 3' ends by a methylguanosine cap and a poly(A) tail respectively. As well as providing termini protection, specific RNA binding proteins associate with both structures which physically interact resulting in the circularisation of the mRNA which facilitates efficient translation (Kahvejian, Svitkin, Sukarieh, M'Boutchou, & Sonenberg, 2005; Wells, Hillner, Vale, & Sachs, 1998). First characterised in yeast and often defined at the 'rate-limiting' step of RNA decay, deadenylation involves two highly conserved complexes, Pan2/Pan3 and CCR4/Not (reviewed in (Wahle & Winkler, 2013), with an additional deadenylase named PARN identified in vertebrates.. The length of the poly(A) tail, which is thought to be at least partially regulated by Pan2/Pan3 mediated trimming (Brown & Sachs, 1998; Yamashita et al., 2005), is a critical determinant of stability (Decker & Parker, 1993; Eckmann, Rammelt, & Wahle, 2011) and can correlate with translational efficiency. (Park, Yi, Kim, Chang, & Kim, 2016). Initial deadenylation is thought to be performed by the Pan2/Pan3 complex which upon their recruitment stimulate the release of the protective Poly(A) Binding Protein (PABP) (Brown & Sachs, 1998; Yamashita, et al., 2005), reviewed in (Wolf & Passmore, 2014). Interestingly, Pan2/Pan3 activity has been shown to be stimulated by PABP and removes a large bulk of the poly(A) tail (which varies in total length between eukaryotic species) but it is inefficient at removing the last few nucleotides which is hypothesised to be because PABP can no longer bind (Brown & Sachs, 1998; Jonas et al., 2014; Lowell, Rudner, & Sachs, 1992).

Following Pan2/Pan3 mediated deadenylation the remaining poly(A) tail is removed by the CCR4/NOT complex (components listed in Table 1) which is recruited to elements in the 3' untranslated region (3' UTR) (reviewed in (Temme, Simonelig, & Wahle, 2014). This ultimately

results in a vulnerable 3’ end which can be attacked by a variety of 3’-5’ exoribonucleases including the exosome complex and Dis3L2. In contrast to the *S. cerevisiae* deadenylase complex where CCR4 is the major deadenylase, Caf1 appears to carry the main deadenylase activity in *Drosophila* cells (Temme et al., 2010), whilst both are required in humans (Maryati, Airhihen, & Winkler, 2015). The major difference between *Drosophila* and mammalian deadenylation is the absence of the additional deadenylase, PARN, from the *Drosophila* genome.

Alternatively, following deadenylation, the transcript can be decapped in a process stimulated by the binding of the Lsm1-7 complex to the oligoadenylated 3’ end (Sharif & Conti, 2013; Tharun, 2009). This both inhibits exosome interaction and stimulates the activity of decapping complexes to remove the 5’ methylguanosine cap allowing exonucleolytic access to the only known cytoplasmic 5’-3’ exoribonuclease, XRN1 (named Pacman in *Drosophila*). XRN1 then degrades the RNA processively from the decapped 5' end through to the 3' end of the RNA.

In addition to exonucleolytic decay, an mRNA can be internally cleaved in endonucleolytic decay. Endonucleolytic cleavage often occurs as a result of quality control mechanisms, including Nonsense-mediated decay (NMD), which functions to prevent translation of error containing mRNA. Cleavage also occurs following highly complementary miRNA binding or in the presence of small interfering RNAs (siRNAs). Internal cleavage of the transcript results in two fragments of the RNA; one containing a vulnerable 3’ end and the other with a vulnerable 5’ end. These fragments are degraded by the exosome complex and XRN1 respectively.

DROSOPHILA MELANOGASTER AS A MODEL ORGANISM

The extensive conservation of the RNA decay machinery means that model organisms are an excellent tool to use to understand how processes involved in RNA stability work and are regulated in the context of developing organisms, rather than an individual cell types growing in culture. The use of the fruit fly has contributed enormously towards our current understanding in a variety of research fields, including RNA stability.

Over the years a number of genetic tools have been made available to *Drosophila* researchers giving an extensive armoury of techniques, helping uncover a number of complex cellular mechanisms (Hales, Korey, Larracuente, & Roberts, 2015). One such elegant tool widely used is the *GAL4-UAS* system which allows both spatial and temporal control over the expression of specific constructs (Duffy, 2002). For example, the expression of a hairpin RNAi to a gene of interest can be restricted to specific tissues and, in the most sensitive of cases, a specific cell type (Pfeiffer et al., 2010). This allows researchers to study the loss of expression effects of even the most critical genes. In addition to the *GAL4-UAS* system, the ground-breaking CRISPR/Cas9 system has been adapted to *Drosophila* work with knock-out or knock-in mutants available in a matter of weeks (Bassett, Tibbit, Ponting, & Liu, 2013).

Extensive conservation of key cellular pathways between humans and *Drosophila* is shown is through evidence that approximately 75% of all disease causing genes in humans have functional *Drosophila* homologues (Reiter, Potocki, Chien, Gribskov, & Bier, 2001). Critically this extensive conservation is seen in the context of RNA stability (summarised in Table 1) (Garneau, Wilusz, & Wilusz, 2007; Kaberdin, Singh, & Lin-Chao, 2011; Newbury, 2006), therefore *Drosophila* provides an

ideal, multicellular model to study these cellular mechanisms providing many advantages over the single cellular models used in RNA stability research. In the following sections we will attempt to outline the advances in our understanding of the role of ribonucleases in regulation of gene expression, their effect on developmental processes in *Drosophila* and how this work has shed light on these pathways in higher organisms.

Table 1: *Drosophila* RNA decay machinery and their human homologues

***Drosophila* Enzyme**	**Human Orthologue**	**Function**
Dcp1	DCP1	Decapping activator
Dcp2	DCP2	Decapping enzyme
Ge-1	EDC4	Decapping activator
Xrn1/Pacman	XRN1	Cytoplasmic 5'-3' RNA decay
Rat1	XRN2	Nuclear 5'-3' RNA decay
Twin	CCR4	Deadenylation
Not1	CNOT1	Deadenylation
Regena	CNOT2	Deadenylation
Not3	CNOT3	Deadenylation
Cnot4	CNOT4	Deadenylation
Pot2	CAF1	Deadenylation
Rcd-1	CAF40	Deadenylation
PAN2	PAN2	Deadenylation
PAN3/CG11486	PAN3	Deadenylation
Dis3/Tazman	DIS3/EXOSC11	3'-5' RNA decay
Dis3L2	DIS3L2	Cytoplasmic 3'-5' RNA decay
Rrp6	RRP6/EXOSC10	Nuclear/Nucleolar 3'-5' RNA decay

5'-3' DECAY BY XRN1/PACMAN

Mechanistic Overview

The *Drosophila* homologue of Xrn1 was first identified in *Drosophila* in 1998 and named Pacman (Till et al., 1998). Since then a number of studies have contributed to our understanding of its role within the cell. In fact, the 5'-3' decay pathway is arguably the most extensively studied area of RNA stability in *Drosophila* (Jones, Zabolotskaya, & Newbury, 2012; Nagarajan, Jones, Newbury, & Green, 2013). XRN1 remains the only known cytoplasmic 5'-3' exoribonuclease and has been shown to be enriched in cellular granules known as Processing bodies (P-bodies). The role of P-bodies within the cell is heavily debated, however, they are known to harbour a number of proteins involved in RNA stability. Their number and size can also be affected by loss of the key decay enzymes. This has been shown in the case of Xrn1/Pacman in *Drosophila* (Zabolotskaya, Grima, Lin, Chou, & Newbury, 2008), where its loss results in an increase in the size of P-bodies; this work is consistent with findings in human HeLa cells where knockdown of XRN1 also results in an increase in P-body size (Lubas et al., 2013).

Interestingly, more recent work has shown that XRN1 is likely to function outside P-bodies as well as within them. For example, Xrn1-mediated decay has also been shown to occur on the ribosome in *Drosophila* S2 cells (Antic, Wolfinger, Skucha, Hosiner, & Dorner, 2015). This work showed Xrn1/Pacman to be present on polysomes and provided evidence that a large proportion of mRNAs are associated with the ribosome during their degradation (Antic, et al., 2015). Work in HeLa cells has also shown the presence of XRN1 on polysomes (Lubas, et al., 2013), suggesting a potential conserved role in co-translational decay, or at least an inherent coupling of translation and RNA decay. This is congruent with recent research showing that codon-optimality can affect RNA decay rate in a number of model organisms. Pioneering data from budding yeast showed that codon optimality is a major determinant of stability (Presnyak et al., 2015). Subsequent research from Bazzini *et al* demonstrated the conservation of this mechanism by showing that transcripts with 'optimal' codons (defined as codons that allow the most efficient translation) in *Drosophila*, mouse, *Xenopus* and zebrafish are far more stable than those with sub-optimal codons (Bazzini et al., 2016). . Additionally, work in *Drosophila* has shown that the decay machinery (i.e. the Ccr4-Not complex) can repress translation by promoting Thor (the *Drosophila* 4E-BP homologue) phosphorylation following insulin stimulation (Okada, Schittenhelm, Straessle, & Hafen, 2015).

For XRN1 to be able to degrade its RNA targets it requires a 5' monophosphate which is created following the removal of the protective 7-methylguanosine cap. Decapping is a critical process in 5'-3' decay and commits capped RNAs to degradation. Detailed structural work by the Izaurralde lab using *Drosophila* Schneider (S2) cells has provided a number of insights into how decapping efficiency is optimised. In *Drosophila,* decapping is performed by a highly conserved complex consisting of Dcp1, Dcp2 and Edc4 (also known as Ge-1). Initial studies elucidated an elegant mechanism through which decapping and decay are linked through physical interaction between the EVH1 domain of Dcp1 and a 'Dcp1-binding motif' in the C-terminal of Xrn1/Pacman (Braun et al., 2012). The functional consequence of this is that Xrn1/Pacman acts as decapping activator for Dcp2. Further support of this role for Xrn1/Pacman comes from phenotypic analysis the in *Drosophila* wing when is overexpressed. These phenotypes are similar to that of *xrn1/pacman* mutants, which could be explained by excess Xrn1/Pacman sequestering Dcp2, resulting in lack of decapping and reduced 5'-3' degradation of target RNAs (Waldron et al., 2015).

The conservation of a direct interaction between XRN1 and the decapping complex was subsequently shown in human HEK293T cells, although the interaction was observed between XRN1 and EDC4 (Braun, et al., 2012). EDC4 has since been shown to function as a key scaffold bringing together DCP2, the catalytic subunit of the complex, and the decapping activator, DCP1 (C. T. Chang, Bercovich, Loh, Jonas, & Izaurralde, 2014). The 'docking' of DCP1 and DCP2 to EDC4 is a critical step for decapping with optimal DCP1-mediated activation of DCP2 occurring on the EDC4 scaffold. This is consistent with early work showing that EDC4 knockdown inhibits decapping in both *Drosophila* and human cells (Eulalio et al., 2007; Fenger-Gron, Fillman, Norrild, & Lykke-Andersen, 2005). Despite the binding partner of XRN1 differing between species, this work has shown the efficient mechanism of decapping, resulting in rapid 5'-3' decay of the substrate in metazoans.

Research conducted in *Drosophila* has also shed light on the mechanisms of NMD (Gatfield & Izaurralde, 2004). Here the authors showed that premature termination codon (PTC) containing transcripts are endonucleolytically cleaved resulting in two fragments: the 3' fragment degraded by Xrn1/Pacman and the 5' fragment degraded by the exosome (Gatfield & Izaurralde, 2004). Since this work, similar mechanisms have been observed in mammalian cells (Eberle, Lykke-Andersen, Mühlemann, & Jensen, 2008). Research from the Izaurralde lab has shown a similar mechanism in RNA interference which, like NMD, results in internal cleavage of the transcript and subsequent degradation; again this has since been shown in human cells (Orban & Izaurralde, 2005). Taken together this work has made critical contributions towards our understanding of mammalian RNA stability pathways.

Xrn1/Pacman deficiencies cause severe phenotypes in *Drosophila*

Research in *Drosophila* has shown the importance of Xrn1/Pacman in maintaining cell and organism viability (Table 2). For example, *xrn1/pacman* null mutants in *Drosophila* show pupal lethality resulting in no adults eclosing (Waldron, et al., 2015). In depth analysis of these mutants revealed increased apoptosis in the larval wing imaginal discs, the tissues responsible for forming the fly wing and thorax, as a result of post-transcriptional upregulation of the pro-apoptotic genes *hid, grim* and *reaper* (Waldron, et al., 2015). Importantly, these phenotypes were rescued by expression of wild-type Xrn1/pacman but not a nuclease-dead version showing the catalytic activity is required to prevent cell death. These null mutants also showed a developmental delay, taking an average of 32 hours longer to pupate, which is likely to be caused by a large post-transcriptional upregulation of the insulin-like peptide, Dilp8 (Jones, Pashler, Towler, Robinson, & Newbury, 2016). The reduction in growth of *xrn1/pacman* mutants in flies is consistent with findings in *S. cerevisiae, Trypanosoma brucei* and *Candida albicans* (An, Lee, & Kim, 2004; J. Kim & Kim, 2002; Larimer & Stevens, 1990; Li et al., 2006; Sinturel, Brechemier-Baey, Kiledjian, Condon, & Benard, 2012).

Further *in vivo* work on Xrn1/Pacman in *Drosophila* has shown that it is required for correct wound healing (Grima et al., 2008) and fertility (Zabolotskaya, et al., 2008). Grima *et al* show how flies carrying hypomorphic mutations in *xrn1/pacman* have a defect in dorsal closure and wound healing which is consistent with growth deficiencies in Xrn1-deficient cells (Grima, et al., 2008). This was proposed to be a result of defects in the c-Jun N-terminal Kinase (JNK) pathway through a genetic interaction with the phosphatase, Puckered. Interestingly, subsequent work has also shown a role for RNA stability in wound healing in mouse myoblasts (Russo et al., 2017). This work revealed that

the loss of the RNA binding protein (RBP) CELF1, which causes destabilisation of its target RNAs, impairs wound healing. Given the similarities of wound healing mechanisms between *Drosophila* and vertebrates further work in the fruit fly may elucidate a role for RNA stability regulation in wound healing. Finally, *xrn1/pacman* hypomorphic mutations have also been shown to cause fertility defects in *Drosophila* with mutants showing morphologically smaller testes together with a reduction in sperm count (Zabolotskaya, et al., 2008). Interestingly, both Xrn1/Pacman and Dis3L2 (discussed below) (Lin et al., 2017) have been shown to be required for correct fertility indicating a key role for RNA decay machinery during spermatogenesis.

Table 2: Phenotypes of *Drosophila* RNA decay mutants

Phenotype	Enzyme Deficiency	Reference
Apoptosis, developmental delay and decreased tissue size	Xrn1/Pacman, Dis3, Rrp40	(Jones et al., 2013; Jones, et al., 2016; Towler et al., 2015; Waldron, et al., 2015)
Defective Wound healing and defects in thorax/dorsal closure	Xrn1/Pacman	(Grima, et al., 2008)
Decreased fertility	Xrn1/Pacman, Dis3L2	(Lin, et al., 2017; Zabolotskaya, et al., 2008)
Lethality	Xrn1/Pacman, Dis3	(Hou, Ruiz, & Andrulis, 2012; Snee et al., 2016; Towler, Jones, Viegas, et al., 2015; Waldron, et al., 2015)
Melanotic masses	Dis3	(Hou, et al., 2012)
Overgrowth in the presence of Ras gain of function mutations	Dis3	(Snee, et al., 2016)
Short mitotic spindles and aneuploidy	Dis3	(Snee, et al., 2016)
Excess posterior follicle cells in stage 5-6 egg chambers	Dis3	(Snee, et al., 2016)
Increased proliferation and tissue size	Dis3L2	(Towler, Jones, Harper, Waldron, & Newbury, 2016)

Endogenous targets of Xrn1/Pacman in *Drosophila*

From the severity of the observed phenotypes it is clear that Xrn1/Pacman is essential to maintain homeostasis, however, surprisingly few transcripts specifically degraded by Xrn1/Pacman have been identified. Studies using hypomorphic and null *xrn1/pacman* mutants have identified a selection of

transcripts that appear to show specific sensitivity to the enzyme. Prior to the advent of RNA-seq, microarrays were used to assess changes to the transcriptome in wing imaginal discs of the hypomorphic mutation *pacman*^{5} (Jones, et al., 2013). Few transcripts showed differential expression, with only 54 genes showing changes in expression >1.5 fold (26 upregulated, 28 downregulated). Of these, *Hsp67Bc*, *CG31477*, *simjang* and *Hsp26* showed post-transcriptional increases suggesting they may be regulated by Xrn1/Pacman. It is important to note that differential expression alone does not mean the above transcripts are direct target of Xrn1/Pacman and further work would be required to separate direct and indirect effects.

Subsequent work using wing imaginal discs from *xrn1/pacman* null mutants lead to the identification of further Xrn1/Pacman sensitive transcripts. For example, three transcripts, encoding the pro-apoptotic proteins Hid, Grim and Reaper, were shown to be post-transcriptionally stabilised in the wing imaginal discs of *xrn1/pacman* null mutants suggesting a direct role for Xrn1/Pacman in regulating pro-apoptotic transcripts (Waldron, et al., 2015). Global RNA-seq analysis in these tissues has further identified *dilp8* and *nplp2* as potential Xrn1/Pacman targets *in vivo* in *Drosophila* (Jones, et al., 2016).

Additional *in vivo* work has identified *E(z)* mRNA as another Xrn1/Pacman target in male larvae, with Mbf1 functioning to protect *E(z)* from Xrn1-mediated decay (Nishioka, Wang, Miyazaki, Soejima, & Hirose, 2018). Complementary work in S2 cells has observed the miRNA targeting components Ago1 and GW182 together with decapped decay intermediates on the ribosome, demonstrating an intriguing role for Xrn1/Pacman in regulating miRNA-directed targets whilst on the ribosome (Antic, et al., 2015). Given this apparent role in miRNA-directed decay, plus its global role in RNA decay it is surprising that these studies reveal surprisingly few transcripts showing differential expression following the loss of Xrn1/Pacman. This may be due to technical factors, tissue specificity, or in many cases due to redundancy in the system where other cytoplasmic decay enzymes, including those discussed below, are also able to degrade Xrn1/Pacman targets in its absence. It is therefore likely that the number of Xrn1 regulated transcripts is much greater than presented here.

CYTOPLASMIC 3'-5' DECAY BY THE EXOSOME.

Mechanistic Overview

In addition to the 5'-3' pathway, RNA can also be attacked and degraded in the 3'-5' direction. However, unlike the 5'-3' pathway where XRN1 is the only known cytoplasmic exoribonuclease with 5'-3' directionality, there are a family of cytoplasmic 3'-5' exoribonucleases. These are highly conserved enzymes with extensive homology with the RNaseII/R family in prokaryotes (Frazao et al., 2006). This 'Dis3' family was first characterised in the yeast *Saccharomyces cerevisiae* where Dis3 was extensively studied and named Rrp44 (Schneider, Anderson, & Tollervey, 2007) and has since been studied in a number of additional models , including *Drosophila melanogaster.*

There are three known members of the Dis3 family in humans: Dis3, Dis3L1 and Dis3L2, with two of these, Dis3 and Dis3L2, found in the *Drosophila* genome. Dis3 was first identified in the *Drosophila* genome by Cairrao *et al*, where it was named Tazman (F. Cairrao, Arraiano, & Newbury, 2005);

however for clarity we will refer to it as Dis3 throughout this review as this is the primary gene name assigned by Flybase. Dis3 and Dis3L1 have been shown to function as catalytic subunits of the exosome complex, an otherwise catalytically inert nine subunit structure, which forms a channel through which the RNA substrate is fed to the active site of Dis3/Dis3L1 for degradation (Kowalinski et al., 2016; Makino, Baumgartner, & Conti, 2013; Wasmuth & Lima, 2012). Together with access via the central channel there is also evidence that a conformational change can allow direct access to the exoribonuclease active site, potentially used by shorter substrates such as miRNAs, which are unable to span the central channel (J. Han & van Hoof, 2016). The interaction of Dis3/Dis3L1 with the exosome has been shown to occur through a CR3 motif and an N-terminal Pilt-N-terminus (PIN) domain in Dis3/Dis3L1 and the core subunits Rrp41 and Rrp45 (Bonneau, Basquin, Ebert, Lorentzen, & Conti, 2009; Malet et al., 2010; Schaeffer, Reis, Johnson, Arraiano, & van Hoof, 2012). In Dis3, the PIN domain also confers endonucleolytic activity (Lebreton, Tomecki, Dziembowski, & Seraphin, 2008; Mamolen, Smith, & Andrulis, 2010; Schaeffer et al., 2009; Schneider, Leung, Brown, & Tollervey, 2009) which gives an additional function potentially allowing decay of more structured substrates that are not fed through the central channel. However, whilst the PIN domain is present in the paralogue, Dis3L1, it is catalytically inactive (Staals et al., 2010; Tomecki et al., 2010). In human cells Dis3 and Dis3L1 show largely distinct cellular compartmentalisation with Dis3 being predominantly nuclear whilst Dis3L1 expression is strictly cytoplasmic (Tomecki, et al., 2010). However, the absence of Dis3L1 in *Drosophila* and *S. cerevisiae* means that Dis3, together with nuclear RNA degradation, compensates and performs the exosome-mediated cytoplasmic 3'-5' RNA decay. Another 3'-5' exoribonuclease, Rrp6, also associates with the exosome core but is only involved in nuclear and nucleolar degradation. Localisation studies show that in *Drosophila* S2 cells Dis3, as in humans, is mainly nuclear but cytoplasmic expression is observed, consistent with Dis3 being is also functional in the cytoplasm in *Drosophila* cells (Graham, Kiss, & Andrulis, 2006). This work also identified the potential of different exosome complex compositions together with the possibility of exosome-independent functions of Dis3 and Rrp6 in *Drosophila* (Graham, Kiss, & Andrulis, 2009). Complementary work in yeast and human cells has demonstrated exosome independent roles for Rrp6 (Callahan & Butler, 2008; Synowsky, van Wijk, Raijmakers, & Heck, 2009; Vallejo, Caparros, & Dominguez, 2011). Although not the subject of this review, it is interesting to note that the nuclear subunit Rrp6 and its human orthologue EXOSC10 has been shown to be required for homologous recombination mediated repair of double strand breaks in both *Drosophila* S2 cells and HeLa cells. This function was at least independent of the core subunit Rrp4 suggesting a role for exosome-independent Rrp6 in DNA damage repair (Marin-Vicente, Domingo-Prim, Eberle, & Visa, 2015). It is therefore possible that the exosome can have different stoichiometries due to accessory factors; however, further work is required to isolate, and identify the functions of the different exosome complexes.

Phenotypic consequences of exosome inactivation

The expression pattern of Dis3 in *Drosophila* was characterised in 2005, where Cairrao *et al* showed differential expression throughout development with the highest expression during embryogenesis, a time where flexibility in gene expression is critical (F. Cairrao, et al., 2005).The importance of Dis3 and its RNA decay activity during *Drosophila* development has been shown in a number of studies (Summarised in Table 2). For example, loss of Dis3 through either mutation or whole organism knockdown results in lethality during the larval stages with the survival to larval stages only possible

due to maternal contribution (Hou, et al., 2012; Snee, et al., 2016; Towler, Jones, Viegas, et al., 2015). This clearly shows how Dis3 is essential to maintain cell and organism viability and is consistent with the pioneering work in *S. cerevisiae* and *S. pombe* together with work in human HEK293 cells where loss of, or mutations in, Dis3 results in a slow growth phenotype (Murakami et al., 2007; Ohkura et al., 1988; Reis et al., 2013; Schaeffer, et al., 2012; Schaeffer & van Hoof, 2011; Smith, Kiss, Turk, Tartakoff, & Andrulis, 2011; Tomecki et al., 2014). This provides an excellent illustration of the advantages of *Drosophila* research, whilst ubiquitous loss of Dis3 is lethal; the *GAL4-UAS* system allows tissue specific depletion which maintains organism viability. For example, in previous work we knocked down Dis3 in the cells specifically fated to form the wing. These cells are fated in the early embryo and are localised to a region of the larval wing imaginal disc termed the wing pouch. Knockdown of Dis3 in these cells resulted in loss of tissue growth as a result of apoptosis of the cells specifically depleted of Dis3. Crucially the organism was viable due to Dis3 expression in all the critical tissues (Towler, Jones, Viegas, et al., 2015).

More recent work by Snee *et al* has provided further mechanistic insights into why such widespread apoptosis was observed (Snee, et al., 2016). This study clearly demonstrates a requirement for Dis3 in cell division with Dis3 mutant neuroblasts showing shorter mitotic spindles than wild-type controls. They also revealed a requirement of the exoribonuclease activity of Dis3's RNB domain for mitotic exit. It is therefore likely that the apoptosis observed in the Dis3 knockdown wing cells (Towler, Jones, Viegas, et al., 2015) is a result of cells encountering a mitotic block (Snee, et al., 2016), which ultimately drives them towards apoptosis. This role for Dis3 in mitosis is consistent with how Dis3 was initially identified in *S. pombe* in a screen for proteins involved in sister chromatid rejoining (Ohkura, et al., 1988), together with subsequent work in both *S. pombe* and *S. cerevisiae* (Kinoshita, Goebl, & Yanagida, 1991; Murakami, et al., 2007; Smith, et al., 2011). Whilst this is yet to be shown in human cells it would be a surprise if Dis3 did not retain this cell-cycle related behaviour. Interestingly, the work by Snee *et al* also showed the first example of post-translational regulation of Dis3 through inhibitory phosphorylation by CDK1 on S786 (Snee, et al., 2016). S786 is a highly conserved and so this may also be a regulatory mechanism observed in human cells which may aid in the understanding of the control of this highly active ribonuclease.

Natural targets of the exosome

It is generally accepted that exosome function can be split into two categories; (1) removal of unwanted RNAs and (2) the 3' end processing and maturation of RNA species. However, compared to Xrn1/Pacman, there has been less work in understanding the direct RNA targets of the exosome in *Drosophila*. Whilst work in *S. cerevisiae* and human cells have shown that Dis3 is involved in the regulation of a vast array of RNA species, including rRNAs, tRNAs, snoRNAs, Promoter Upstream Transcripts (PROMPTs) and Cryptic Unstable Transcripts (CUTs) (Allmang et al., 1999; Gudipati et al., 2012; Kadaba et al., 2004; Mitchell, Petfalski, Shevchenko, Mann, & Tollervey, 1997; Preker et al., 2008; Schneider, et al., 2007; Schneider, Kudla, Wlotzka, Tuck, & Tollervey, 2012; Tomecki, et al., 2014; Wyers et al., 2005), this is a body of work largely absent in *Drosophila*. This is likely due to the traditional method of identifying ribonuclease-sensitive transcripts, through enzyme depletion and analysis of differential expression. As previously mentioned, loss of Dis3 throughout the organism is lethal and therefore more targeted approaches, such as that performed in wing imaginal discs (Towler, Jones, Viegas, et al., 2015), are required.

A few studies have attempted to identify Dis3/Exosome sensitive transcripts in *Drosophila*. Work conducted in *Drosophila* S2 cells using microarrays reported that most affected transcripts increased in expression following exosome depletion which one may expect given the exosome's primary function in RNA degradation (Kiss & Andrulis, 2010). One observation in exosome deficient S2 cells was that most stabilised mRNAs had longer than average UTRs, particularly 3' UTRs (Kiss & Andrulis, 2010) which may be due to specific 'instability elements' such are AU rich elements or miRNA binding sites. This provides further evidence of the importance of the UTRs in regulating RNA stability. Within this data the most common GO term for Dis3 or Rrp6 sensitive transcripts was "Cell Cycle", which is concurrent with the *in vivo* phenotypic observations following Dis3 depletion (Snee, et al., 2016; Towler, Jones, Viegas, et al., 2015).

Two subsequent studies have used global RNA-sequencing to assess transcriptome changes following knockdown of Dis3 in the developing fly. Hou *et al* assessed changes in expression across the whole organism at various developmental stages (Hou, et al., 2012), whereas Towler *et al* used a targeted approach investigating the cytoplasmic function of the exosome on regulating miRNA stability within the wing imaginal disc (Towler, Jones, Viegas, et al., 2015). Surprisingly, during early development, (embryogenesis and the first larval stage), Dis3 depletion results in a general downregulation of transcripts rather than an upregulation as one may expect considering the degradative role of the exosome. This would suggest that the effects observed are either indirect, or that during early development the exosome's role in RNA maturation may be more apparent, such as that previously observed for 5.8S rRNA maturation (Mitchell, et al., 1997; Schneider, et al., 2012). A deficiency in such pathways may also explain the growth deficiencies, as discussed previously.

To date only *miR-252-5p* (Towler, Jones, Viegas, et al., 2015)*, kruppel, hunchback and CG2011* (Hou, et al., 2012) have been named and discussed as exosome-sensitive transcripts in *Drosophila* with *miR-252-5p* the only characterised transcript in *Drosophila* to show a post-transcriptional regulation. However, being a target of the cytoplasmic exosome, the difference between the catalytic subunits (Dis3 in *Drosophila* vs Dis3L1 in humans) means there may be key differences observed. Further investigation into these datasets may elucidate further exosome targets and aid in the understanding of the phenotypes observed following the loss of Dis3.

CYTOPLASMIC 3'-5' DECAY BY DIS3L2

Mechanistic Overview

Dis3L2, a paralogue of Dis3, is the other major exoribonuclease responsible for 3'-5' decay in the cytoplasm in *Drosophila*. Interestingly, Dis3L2 is not found in the genome of *S. cerevisiae*, the historical workhorse of RNA stability research, meaning its existence has only recently come to light. Like Dis3, Dis3L2 is a highly processive enzyme, however, unlike its paralogue, it acts independently of the exosome complex as it lacks the N-terminal PIN domain (Lubas, et al., 2013; Malecki et al., 2013). The absence of the PIN domain means that Dis3L2 is most similar to the prokaryotic RNaseII/R. Work in human and yeast cells has shown that Dis3L2 shows a striking preference for RNAs carrying a polyU tail which appears to act as a decay signal for Dis3L2 (H. M. Chang, Triboulet, Thornton, & Gregory, 2013; Malecki, et al., 2013; Ustianenko et al., 2013). Structural work on mouse Dis3L2 has shown that this preference is due to the composition of the central channel leading to the active site (Faehnle, Walleshauser, & Joshua-Tor, 2014).

To date, *Drosophila* is the only organism in which a direct binding partner of Dis3L2 has been identified. Work performed in S2 cells in two independent laboratories has recently shown a direct interaction between Dis3L2 and a terminal uridylyl transferase (TUTase) named Tailor through N-terminal domains in both proteins (Lin, et al., 2017; Reimao-Pinto et al., 2016). Mutational studies have shown that the interaction occurs through a domain of unknown function (DUF1439) in Tailor and a coiled-coil domain in Dis3L2. These domains are not conserved, which is consistent with work in human HEK293 cells where immunoprecipitation experiments failed to identify a direct binding partner of Dis3L2 (Lubas, et al., 2013). While these two studies show a direct interaction between Dis3L2 N-terminal domains and the TUTase (Tailor) there were two discrepancies. The first of these is the effect of Tailor depletion (by CRISPR/Cas9) on Dis3L2 levels demonstrating that loss of Tailor results in a subsequent destabilising of Dis3L2 in both S2 cells and *in vivo* ovary lysates (Reimao-Pinto, et al., 2016). In contrast, Lin et al presented data showing Tailor depletion (by RNAi) has no effect on the levels of endogenous Dis3L2 (Lin, et al., 2017). The second discrepancy is in the colocalisation of the two proteins. Whilst both Tailor and Dis3L2 were shown to be cytoplasmic in S2 cells, as seen previously in *S. pombe* and human cells (Lubas, et al., 2013; Malecki, et al., 2013), Reimao-Pinto *et al* saw extensive colocalisation, whereas Lin *et al* also observed a number of independent foci suggesting the two proteins may also have independent functions. It is also possible that their localisation varies depending on cell physiology; for instance, work in *S. pombe* has shown that Dis3L2 localisation alters upon glucose starvation (Malecki, et al., 2013). These discrepancies could be explained by technical differences including the methods of depletion and detection but further work is clearly required to understand the cooperation between Tailor and Dis3L2 *in vivo*.

Phenotypes resulting from loss of Dis3L2

Despite mechanistic differences, these papers together with a study from our lab have significantly contributed to our understanding of Dis3L2 mediated decay in *Drosophila* (Lin, et al., 2017; Reimao-Pinto, et al., 2016; Towler, et al., 2016). They have identified key roles for this conserved exoribonuclease in fundamental cellular and developmental processes (summarised in Table 2). Lin *et al* clearly show that flies lacking Dis3L2 display strong fertility defects that are more profound in males than females (Lin, et al., 2017). In this work the authors demonstrate that Dis3L2 is required for spermatogenesis with mutants showing seminal vesicles (the storage vessel for mature sperm cells in *Drosophila* testes) completely devoid of mature sperm cells.

In addition to a role for Dis3L2 in spermatogenesis our research has shown that Dis3L2 is required to control developmental proliferation in the wing imaginal disc (Towler, et al., 2016). This work has revealed that the loss of Dis3L2 in the *Drosophila* wing imaginal discs results in an increase in proliferation within these tissues. This ultimately results in an increase in imaginal disc area resulting in an increase of adult wing area as a result of hyperplasia. Whilst there remains a possibility of tissue specific effects, these results are concurrent with work in HeLa cells. Whilst HeLa cells are perhaps not the most physiologically relevant cell line, Dis3L2 knockdown was also shown to increase cell number with a selection of cell-cycle related transcripts affected (Astuti et al., 2012). The phenotypes are also strikingly similar to two human overgrowth disorders, Perlman Syndrome

and Wilms' Tumour, which have been associated with Dis3L2 mutations suggesting a crucial and conserved function (Astuti, et al., 2012; Morris, Astuti, & Maher, 2013).

Identification of Dis3L2 RNA targets

Previous work from human and yeast cells has shown that Dis3L2 preferentially degrades RNAs that have uridine residues added to their 3' end (H. M. Chang, et al., 2013; Malecki, et al., 2013; Thomas et al., 2015) such as the precursor miRNA *pre-let-7a* (H. M. Chang, et al., 2013). Together with the phenotypic analysis of Dis3L2-deficient flies, three subsequent studies have contributed towards the identification of Dis3L2-sensitive transcripts in *Drosophila* (Lin, et al., 2017; Reimao-Pinto, et al., 2016; Towler, et al., 2016). A direct approach has been performed in S2 cells where a catalytically inactive version of Dis3L2 was immunoprecipitated and the co-precipitating RNA was subsequently subjected to RNA-sequencing. In this work, a catalytically inactive version of the ribonuclease is required, as in a wild-type condition the process of degradation often occurs too rapidly to detect enzyme-associated RNAs (Reimao-Pinto, et al., 2016). This work identified a number of 'direct' Dis3L2 targets in *Drosophila* tissue culture cells; however, the vast majority of RNAs co-precipitating with Dis3L2 were unprocessed 5S rRNA transcripts This suggests a role for Dis3L2 in rRNA maturation, a function previously shown for the other 3'-5' ribonucleases Dis3 and Rrp6 (Mitchell, et al., 1997; Schneider, et al., 2012). Interestingly this unprocessed transcript was only observed in cells carrying the catalytically dead mutation and not in cells with a simple depletion, suggesting a redundant function, presumably with either Dis3 or Rrp6. This also emphasises the importance of experimental design in identifying ribonuclease targets. Together with unprocessed 5S rRNA extended versions of *RNase MRP:RNA*, and a pool of tRNAs were also observed, showing the role of Dis3L2 in RNA maturation is not confined to 5S rRNA. Importantly, the authors showed this function was not solely in tissue culture cells but also observed in whole male flies that either expressed the catalytic mutant or were depleted for Dis3L2 showing a more global, specific function for Dis3L2.

In addition to the proposed maturation function the authors also present a role for Dis3L2 in the quality control of RNA pol III transcripts which is similar to that previously observed in human cells (Pirouz, Du, Munafo, & Gregory, 2016). The majority of Dis3L2-associated RNAs were ncRNAs such as tRNAs, snRNAs and snoRNAs with limited association of mRNAs. Crucially, similar *in vivo* observations were made by Lin and colleagues in the *Drosophila* testes where proportionally many more lncRNAs were differentially expressed than mRNAs in Dis3L2 knockout tissues (Lin, et al., 2017). Here, the authors identify a pool of lncRNAs that appear sensitive to Dis3L2 mediated repression. In addition to these ncRNAs, a study from our lab identified a pool of mRNAs that also appear to show sensitivity to Dis3L2 (Towler, et al., 2016). In line with the observations above we identified a limited number of polyA(+) RNAs (239 or 2.7% of detected transcripts showing specific Dis3L2 sensitivity in imaginal discs. Within these a cation channel (*pyrexia*) and a predicted transcription factor (*CG2678*) showed a post-transcriptional increase in expression in Dis3L2 knockdown wing imaginal discs suggesting they may be directly regulated by the enzyme.

All three of the above studies represent the diversity of potential Dis3L2 substrates and therefore indicate a number of roles for Dis3L2 in maintaining homeostasis, including RNA maturation, quality control and general RNA decay. This collection of work also demonstrates the importance of *in vivo* analysis as the use of different tissues/cells permits the identification of both tissue specific and

'global' Dis3L2 targets. Undoubtedly, further work is required to build upon these studies, develop the pool of known Dis3L2 targets, and ascertain the pathways controlled by this exoribonuclease.

RNA TARGETING – REGULATING THE REGULATORS

The key question in the RNA stability field is how the decay enzymes are targeted to their specific substrates at specific stages of a cells life. An extensive battery of work in *Drosophila* has begun to shed some light on these fundamental and conserved mechanisms which appear to be a combination of *cis* and *trans* acting factors many of which are associated or bind within the 3' UTR. 3' UTR length has a strong influence on RNA stability with mRNAs with long (>1000nt) or short (<100nt) 3' UTRs showing lower expression than those with an intermediate length (Spasic et al., 2012). Our current understanding of these targeting mechanisms in *Drosophila* will be discussed further below and are shown in Figure 1.

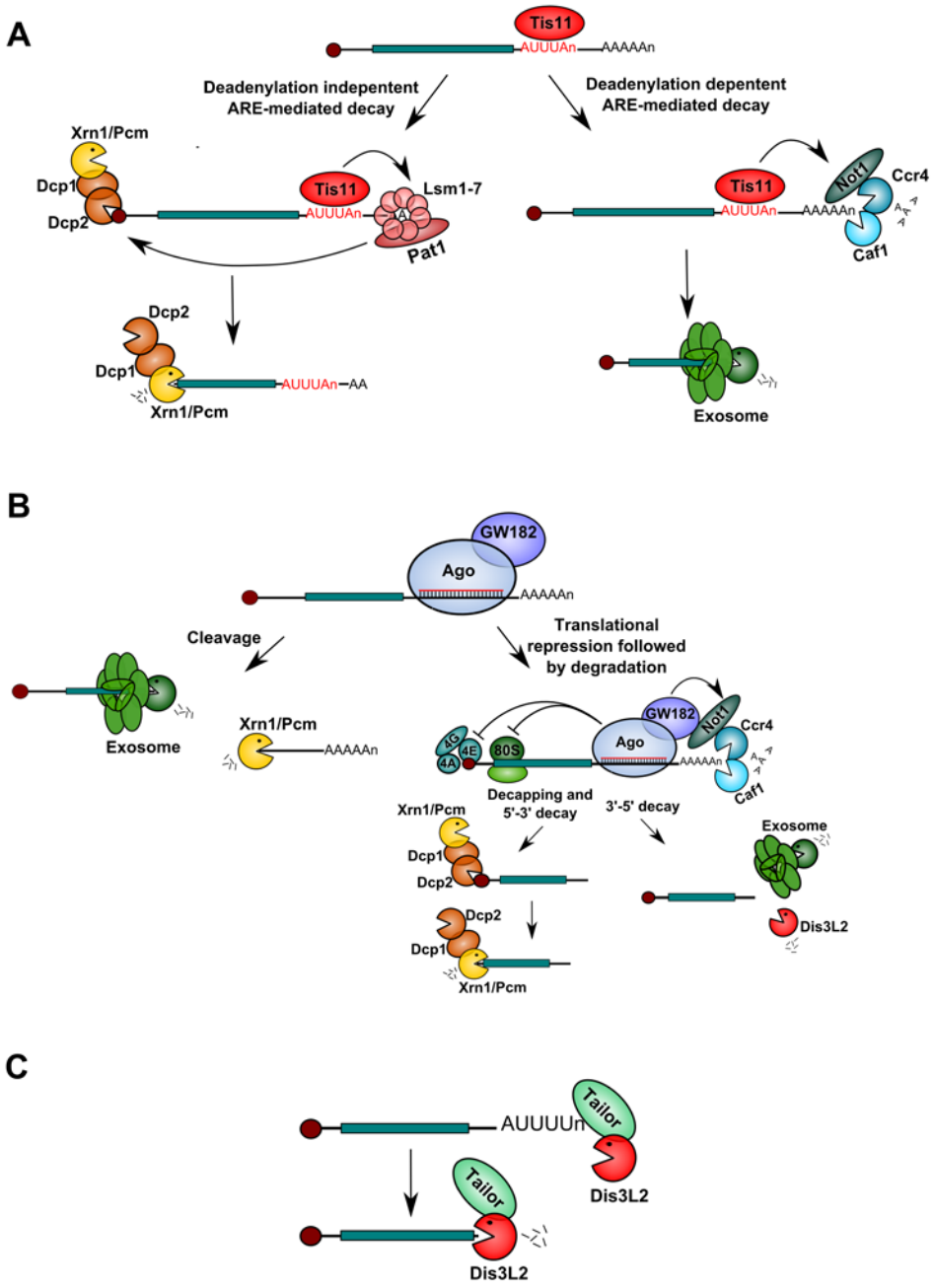


Figure 1: Methods of targeting decay. A) AU rich elements (AREs) usually within the 3' UTR recruit specific RNA binding proteins (*Drosophila* TTP homology Tis11 shown). ARE binding proteins subsequently promote 3'-5' decay by the exosome where in *Drosophila* Dis3/Tazman (named Rrp44 in *S. cerevisiae*) provides catalytic activity or 5'-3' decay by XRN1/Pacman (Pcm). The direction of decay can be transcript and protein specific. **B)** miRNAs direct the RNA Induced Silencing Complex (RISC) containing either Ago1 or Ago2 to specific target sites, usually in the 3' UTR, and can promote translational repression at the stages of initiation or elongation depending on the nature of the Ago protein. When extensive complementarity is present between a miRNA and its target Ago2-RISC directs cleavage of the target RNA. Extended repression can also result in mRNA degradation through classical decay pathways. **C)** An mRNA can be targeted for decay following nucleotide additions to the 3' end. Uridylation by a *Drosophila* Terminal Uridylyl Transferase (TUTase) named Tailor is depicted. This primes the RNA for Dis3L2 mediated decay.

AU rich elements

There are a number of 'stability elements' present in an RNA molecule which function to confer its stability, many residing in the 5' and 3' untranslated regions. Perhaps one of the earliest characterised examples of these are AU rich elements (AREs) which are stretches of Adenine and Uracil nucleotides (AUUUA being the most common motif) within the 3' UTR of a specific subset of mRNAs. In 1995 Chen and Shyu grouped AREs into three distinct classes based on the motifs and these classifications remain widely used (Chen & Shyu, 1995). AREs have been observed within the 3' UTR of 5-8% of mammalian mRNAs and are often found in those with shorter half-lives such as those encoding for oncogenes and cytokines (T Bakheet, Frevel, Williams, Greer, & Khabar, 2001; T. Bakheet, Hitti, & Khabar, 2018; T. Bakheet, Williams, & Khabar, 2006; Schoenberg & Maquat, 2012). AREs recruit a specific set of RNA binding proteins, in most cases this RNA-protein interaction promotes the decay of the bound RNA, through both 5'-3' and 3'-5' decay pathways (Murray & Schoenberg, 2007) (Figure 1A), although some examples do exist of ARE-binding proteins promoting stability and translation (Abdelmohsen & Gorospe, 2010; Peng, Chen, Xu, & Shyu, 1998).

ARE-mediated decay is a key mechanism of regulating stability in *Drosophila* as well as in mice and humans. It has been reported that up to 16% of *Drosophila* mRNAs contain the consensus ARE motif within their 3' UTR (F Cairrao, Halees, Khabar, Morello, & Vanzo, 2009). Interestingly, this *in silico* screen also demonstrated that ARE containing 3' UTRs were 2.5 times longer than those without. A subsequent study by Spasic *et al* using *Drosophila* haemocytes (SL2 cells) has built upon this knowledge, resulting in the algorithm ARE*Score*, a publically available tool which can be used to assess AREs within *Drosophila* transcripts (Spasic, et al., 2012). Importantly this study demonstrated that like human 3' UTRs, *Drosophila* 3'UTRs are significantly enriched for AREs, and they appear to be a global mechanism regulating RNA stability, providing further evidence that *Drosophila* are indeed an excellent model to study ARE biology.

Within *Drosophila,* Tis11 has been by far the best characterised ARE-binding protein. The Tristetraprolin (TTP) homologue regulates the expression of a subset of RNAs including *Vir1*, *cecA1* and *branchless* (F Cairrao, et al., 2009; Spasic, et al., 2012; Vindry et al., 2012; Wei et al., 2009) with the ARE-mediated regulation of *cecA1* stability confirmed *in vivo* in the fat body (F Cairrao, et al., 2009). Consistent with a role for a coupling of translation and decay as discussed previously, Tis11 has been shown to promote deadenylation of polysomal *cecA1* (Vindry, et al., 2012). Crucially, the conservation of this mechanism has been confirmed by work showing that mammalian TTP compensates for Tis11 depletion in S2 cells (Wei, et al., 2009). An outstanding question in ARE biology remains the polarity of degradation induced by ARE-; for instance, the AREs in the *IL-3* 3' UTR specifically direct 3'-5' mediated decay in a Tis11 dependent manner, as depletion of *pcm* has no effect on reporter expression. This is congruent with previous work in human cells showing that AREs in different 3' UTRs direct the decay machinery in different manners (Murray & Schoenberg, 2007). Further work is therefore required to understand how certain AREs within specific 3' UTRs direct the decay machinery. One may hypothesise that this is likely to be due to the specific ARE binding proteins and their cofactors.

miRNA mediated decay

As well as AREs, the 3'UTR contains other regulatory sequences on which another key family of regulators act. These regulators are microRNAs (miRNAs), small non-coding RNAs which are 18-23 nucleotides in length, and largely function to negatively regulate the expression of their targets. They bind to complementary sequences, normally in the 3' UTR, of mRNAs of which the key region of interaction is between nucleotides 2-8 of the miRNA, a region known as the seed. miRNAs function as the guide sequence targeting the RNA Induced Silencing Complex (RISC) to target mRNAs. Work in both *Drosophila* cell lines, embryo lysates and adults flies has increased our understanding of miRNA targeting. For example, Tomari *et al* demonstrated that mature miRNAs can be loaded into one of two RISC's containing either Ago1 or Ago2 which is regulated by the structure of the small RNA duplex; with most miRNAs being loaded into Ago1-RISC as a result of containing central bulges in the pre-miRNA (Tomari, Du, & Zamore, 2007).

Although the results of miRNA-target interactions are normally a reduction in expression, the mechanism of downregulation varies depending on complementarity. For example, those few miRNAs incorporated into Ago2-RISC require extensive complementarity with their targets to generate repression and ultimately target cleavage (Forstemann, Horwich, Wee, Tomari, & Zamore, 2007). This results in two RNA fragments with vulnerable 3' and 5' ends which are degraded by the exosome and Xrn1/Pacman respectively Figure 1B. Alternatively, mature miRNAs originating from duplexes containing central mismatches, which represents the majority of miRNAs in *Drosophila*, are loaded into Ago1-RISC. Ago1-RISC is able to tolerate mismatches between miRNAs and their targets; however, due to Ago1 having very poor slicing activity the major method of repression occurs through translational repression (Forstemann, et al., 2007).

This process of miRNA-mediated repression is highly conserved in all eukaryotes. Interestingly, work initially conducted in *Drosophila* cells showed that GW182 and the decapping complex were required for miRNA-mediated gene silencing (Rehwinkel, Behm-Ansmant, Gatfield, & Izaurralde, 2005). Subsequent work in *Drosophila* has shown that Ago1 mediated translational repression, but not Ago2 mediated repression, requires GW182 and occurs through promoting shortening of the polyA tail resulting in a reduction in translational efficiency. This work also demonstrated that although Ago2-RISC normally cleaves its target RNAs it is also able to promote translational repression through competing with eIF4G for the binding of eIF4E (Iwasaki, Kawamata, & Tomari, 2009).

The importance of miRNA-mediated regulation in *Drosophila* has been demonstrated in a number of studies. For example, in a global approach using the *GAL4-UAS* system, Schertel *et al* overexpressed 180 miRNAs in the whole fly (*act5c-GAL4*), the wing (*MS1096-GAL4*) and the eye (*ey-GAL4*) and examined the resulting phenotypes (Schertel, Rutishauser, Förstemann, & Basler, 2012). The results varied with some giving very mild phenotypes and others resulting in lethality when overexpressed ubiquitously. In total, 78 of the 180 miRNAs overexpressed gave an observable phenotype in at least one of the screens, showing the overall importance of this arm of post-transcriptional regulation. The authors also observed a clear correlation between miRNA expression (Berezikov, 2011) and the observation of a phenotype, with 73% of the highest expressed (49) miRNAs presenting a phenotype when overexpressed.

These approaches have identified a number of miRNAs that are involved in regulating specific pathways through conserved mechanisms. Two separate studies have identified potentially conserved roles for miRNA-mediated regulation of Notch signalling in *Drosophila.* For example, Schertel *et al* demonstrated a role for *miR-92a* in regulating wing hair development through regulating the mRNA encoding Shavenoid (Schertel, et al., 2012) whilst Vallejo *et al* show that *miR-8* plays a critical role in regulating the Notch ligand Serrate (Vallejo, et al., 2011). Other similar studies have observed role for *miR-14* as a regulator of Hedgehog signalling (K. Kim, Vinayagam, & Perrimon, 2014), *miR-9a* in regulating body growth through suppressing sNPFR1/NPYR (regulators of insulin signalling) (Suh et al., 2015), and finally *miR-190* in hypoxia (De Lella Ezcurra et al., 2016). With the exception of *miR-14*, all these miRNAs are conserved with all three pathways implicated in human disease. Vallejo *et al* also showed that overexpression of the *miR-8/200* family can inhibit proliferation of human prostate cancer cells (Vallejo, et al., 2011). Therefore, from these studies, it is clear how miRNA screening in *Drosophila* could have particular importance in understanding human homeostasis and disease.

Despite these extensive contributions there is still much to be learnt about miRNA-mediated regulation. Currently, much work is going into improving miRNA target prediction algorithms, which, while useful, are variable and require experimental validation. Additionally, very little is known about how the stability of miRNAs themselves are regulated (reviewed in (Towler, Jones, & Newbury, 2015). It has recently been shown that when miRNA targeting promotes target decay, the miRNA itself can also be degraded in a process known as target-directed miRNA decay (TDMD) This process was first identified in *Drosophila* (Ameres et al., 2010) and subsequently confirmed in mammalian systems (Baccarini et al., 2011; de la Mata et al., 2015; Haas et al., 2016). Additional modifications in the regulation of miRNA, along with other RNA species, will be discussed below.

3' end tailing targeting decay

Following the discovery of uridylation in yeast, and subsequently in human cells, its presence in *Drosophila* remained elusive. Whilst Cid1 (Rissland, Mikulasova, & Norbury, 2007) and TUT4/TUT7 (Heo et al., 2009; Lim et al., 2014) in *S. pombe* and humans respectively are responsible for 3' uridylation, the *Drosophila* nucleotidyl transferase responsible for 3' uridylation remained unknown. However, recent work has identified Tailor as a terminal uridylyl transferase (TUTase) responsible for 3' uridylation of pre-miRNAs derived from introns, termed 'Mirtrons', during their biogenesis in *Drosophila* S2 cells, whole flies and ovaries (Bortolamiol-Becet et al., 2015; Reimao-Pinto et al., 2015).Tailor-mediated pre-miRNA uridylation prevents Dicer mediated hairpin cleavage thereby preventing mature miRNA biogenesis (Bortolamiol-Becet, et al., 2015; Reimao-Pinto, et al., 2015). This mechanism is strikingly similar to the first example of the interplay between uridylation and Dis3L2-mediated decay, shown in human HeLa cells for *pre-let-7a* regulation (H. M. Chang, et al., 2013; Ustianenko, et al., 2013),. A combination of high throughput and targeted approaches in both these studies has shown that the depletion of Tailor results in the modest stabilisation of many mature miRNAs, particularly the 3p species of intron derived mature miRNAs with a clear reduction in 3' U additions. This is consistent with a global screen performed in 2011 which saw a bias for uridylation of the 3p arm vs the 5p (Berezikov, 2011). There did appear to be a few examples where Tailor mediated uridylation promoted processing, such as that presented for *pre-bantam*, a

canonical miRNA which may imply different Tailor-mediated mechanisms depending on the genomic origin of the miRNA (Reimao-Pinto, et al., 2015).

Interestingly, the selective uridylation of pre-miRNAs derived from introns appears to be dependent on the 3' nucleotides, due to the fact that Tailor shows an affinity for 3' G or AG nucleotides (Bortolamiol-Becet, et al., 2015; Reimao-Pinto, et al., 2015). With 3' AG being a well characterised splice acceptor site, it is possible that this poses a mechanism through which Mirtron levels are controlled. This hypothesis is strengthened by the observation that although 'canonical' miRNAs that originate from their own independent gene can carry a 3' G or AG (Bortolamiol-Becet, et al., 2015) they are significantly less enriched when compared to Mirtron precursors (Reimao-Pinto, et al., 2015).

Together with tailing of pre-miRNAs, pioneering work in *Drosophila* also showed examples of 3' tailing or trimming of mature miRNAs. Ameres *et al* used *Drosophila* S2 cells together with embryo lysates to show that miRNAs incorporated in Ago1-RISC as susceptible to 3' tailing and trimming when extensive complementarity is present. Here the authors hypothesised that extensive 3' complementarity results in the release of the 3' end from the PAZ domain therefore making it accessible to tailing and trimming enzymes. Interestingly, this phenomenon was only observed for Ago1-RISC bound miRNAs as Ago2-RISC miRNAs contain a 2'-O-methyl group at the 3' end, placed by Hen1, which prevents the enzyme access to the miRNA termini (Ameres, et al., 2010). A subsequent study in *Drosophila* identified the 3'-5' exoribonuclease Nibbler as the enzyme responsible for mature miRNA trimming (B. W. Han, Hung, Weng, Zamore, & Ameres, 2011; Liu et al., 2011) . Tailing and trimming of mature miRNAs has since been shown to be a conserved mechanism in a number of eukaryotes some examples of which are discussed further below (Bitetti et al., 2018; Haas, et al., 2016; Juvvuna, Khandelia, Lee, & Makeyev, 2012; Modepalli & Moran, 2017).

The importance of uridylation in maintaining homeostasis is perhaps best described in *Drosophila* where Tailor null mutant males, and to a lesser extent females, show reduced fertility (Bortolamiol-Becet, et al., 2015; Reimao-Pinto, et al., 2015). This, taken with the evidence discussed previously (Lin, et al., 2017) suggests a uridylation-mediated Dis3L2 decay pathway which is critical in maintaining *Drosophila* fertility. It is possible that this control is achieved through the regulation of pools of Mirtrons or lncRNAs which have been shown to be sensitive to the Tailor/Dis3L2 axis of decay (Lin, et al., 2017; Reimao-Pinto, et al., 2015; Reimao-Pinto, et al., 2016). Although Tailor activity is clearly important one significant factor to note is that pre-miRNA uridylation was still observed in Tailor null cells, therefore there must be other TUTases in *Drosophila* that are at least partially redundant with Tailor in terms of pre-miRNA uridylation. A recent study by Modepalli and Moran has explored the evolution of miRNA tailing in metazoa and presents a good starting point for the identification of these and other elusive TUTases (Modepalli & Moran, 2017).

In addition to uridylation, 3' adenylation was also observed in both studies discussed here, which was unaffected by Tailor depletion (Reimao-Pinto, et al., 2015), utilising a mechanism similar to that observed in *Drosophila* embryos (Lee et al., 2014). Here it was shown how another terminal nucleotidylyl transferase (TNTase), Wispy, is responsible for targeting maternally provided mature miRNAs for decay during the maternal-zygotic transition. Curiously, Wispy expression is absent from S2 cells, one of the models used in the studies by Reimao-Pinto *et al* and Bortolamio-Becet *et al*,

therefore there must be other TNTases that also modulate stability by 3' tailing which remain elusive. miRNA adenylation has also been described in human cells (Burroughs et al., 2010; Katoh et al., 2009; Wyman et al., 2011), such as that shown for *miR-122* where the addition of a single A to the 3' end results in miRNA stabilisation (Katoh, et al., 2009), suggesting a conserved mechanism.

Uridylation was first shown to be a mechanism for targeting histone mRNAs (which do not have poly(A) tails) for decay by both XRN1 and the exosome in HeLa cells (Mullen & Marzluff, 2008). However, since then it has been shown that Dis3L2 shows a striking preference for degrading uridylated substrates. This preference is due to the composition of its internal channel leading to the active site (Faehnle, et al., 2014). It would be of great interest to investigate how the method of tailing for these substrates applies across the *Drosophila* genome, including mRNAs and other ncRNAs as has been observed in humans and *S. pombe* (Malecki, et al., 2013; Thomas, et al., 2015). It is clear therefore that 3' tailing (including uridylation as reviewed in (De Almeida, Scheer, Zuber, & Gagliardi, 2018) has a critical role in regulating RNA stability and further characterisation of the enzymes responsible, particularly in multicellular models such as *Drosophila*, would be of great interest.

USING *DROSOPHILA* RNA STABILITY RESEARCH TO UNDERSTAND HUMAN DISEASE

A number of factors involved in regulating RNA stability, including the exoribonucleases themselves, have been implicated in human disease (summarised in Table 3 and reviewed in (Pashler, Towler, Jones, & Newbury, 2016). Work in *Drosophila* has contributed to our understanding of how these factors contribute to disease (Snee, et al., 2016). For example, Dis3 is frequently mutated in Multiple Myeloma (MM) (M. A. Chapman et al., 2011), Acute Myeloid Leukaemia (AML) (Ding et al., 2012) and Chronic Lymphocytic Leukaemia (Ng et al., 2007). Subsequent biochemical work has shown that some of the mutations frequently observed in MM result in the loss of function of Dis3 activity (Tomecki, et al., 2014). Considering the above evidence from *Drosophila* together with that from yeast where the loss of Dis3 results in a reduction in growth and lethality, this seems counter-intuitive. However, recent work has shown the potential of *Drosophila* RNA stability research in understanding the role of Dis3 in these diseases (Snee, et al., 2016). In this work, Snee and colleagues elegantly show that Dis3 and Ras genetically interact to control tissue growth. They clearly demonstrate that when Dis3 is lost alongside the overexpression of an activated Ras, extensive tissue overgrowth is observed at greater severity than when an activated Ras is overexpressed alone. The loss of Dis3 alleviates the G2/M delay observed when Ras is overexpressed alone; causing a release that facilitates uncontrolled proliferation. Ras is frequently overexpressed in MM cells (Steinbrunn et al., 2011) and therefore this work elegantly provides the first mechanistic insight into how Dis3 loss of function mutations may elicit a growth advantage to MM cells.

In addition to the implication of Dis3 in human disease, genomic deletions of Dis3L2 have been associated with two overgrowth conditions: Perlman syndrome and Wilms' tumour (Astuti, et al., 2012; Morris, et al., 2013). Recent work has shown that the knockdown of Dis3L2 in the wing imaginal disc also results in tissue overgrowth as a result of increased cell number, therefore phenocopying the symptoms of the human disorders (Towler, et al., 2016). This has provided the first non-human animal model which may facilitate increased understanding of the conserved pathways affected in these diseases.

Together with a role for RNA stability proteins in cancer, RNAi screens in *Drosophila* DL1 cells have identified a conserved role for a number of stability mediators in the antiviral response. In two separate screens depletion of both the 3'-5' and 5'-3' machinery increased the cells susceptibility to infection (Hopkins et al., 2013; Molleston et al., 2016). In the case of the 3'-5' pathway depletion of the exosome components Dis3, Rrp6, Rrp4, Rrp4, together with members of the TRAMP complex Mtr4 and Zcchc7 in both DL1 cells and the adult fat body (with the exception of Dis3 in the fat body) results in an increase in RNA viral infection. The functional conservation of this antiviral mechanism was confirmed in human U2OS cells (Molleston, et al., 2016). Interestingly, the decapping enzyme Dcp2, together with decapping activators Ddx6 and Lsm7 were previously shown to prevent viral replication with their depletion facilitating viral transcription without affecting stability (Hopkins, et al., 2013). Taken with work demonstrating a neat mechanism through which flaviviruses block XRN1 activity in human cells (E. G. Chapman, Moon, Wilusz, & Kieft, 2014; Moon et al., 2015), suggests an inherent antiviral mechanism for the decay machinery against RNA viruses. For further information on how *Drosophila* are used in medical research see (Pandey & Nichols, 2011).

Table 3: RNA decay machinery in disease

Associated Protein	Nature of Mutation	Condition	Reference
Dis3	Loss of function	Multiple Myeloma, Acute Myeloid Leukaemia, Chronic Lymphocytic Leukaemia	(M. A. Chapman, et al., 2011; Ding, et al., 2012; Ng, et al., 2007)
Dis3	Overexpression	Colorectal carcinoma, Nodular melanoma	(de Groen et al., 2014; Rose et al., 2011)
Dis3L2	Loss of function through genomic deletion	Perlman syndrome, Wilms' tumour	(Astuti, et al., 2012)
XRN1	Reduced levels in cell lines and patients compared to control	Osteosarcoma	(Zhang et al., 2002)
XRN1	Mechanistically inhibited by Viruses	Flaviviral infection	(E. G. Chapman, et al., 2014; Moon, et al., 2015)
EXOSC3	Gly31Ala, Val80Phe, Asp132Ala	Pontocerebellar hypoplasia type 1B	(Eggens et al., 2014; Wan et al., 2012)
EXOSC2	Missense	Retinitis Pigmentosa, hearing loss, mild intellectual disability	(Di Donato et al., 2016)
EXOSC8	Homozygous missense, Ser272Thr,	Psychomotor deficit, cerebellar hypoplasia, Spinal	(Boczonadi et al., 2014)

	Ala2Val,	muscular atrophy

LIMITATIONS OF *DROSOPHILA* WORK

Whilst *Drosophila* does provide an excellent model system to study RNA stability there are some limitations which must be considered. For example, whilst reduced redundancy and complexity in many pathways could be seen as a great benefit, it could also be interpreted as a limitation as it is possible that a phenomenon critical in *Drosophila* may be less so in humans due to increased redundancy. For example, the *Drosophila* genome does not encode the deadenylase PARN meaning the specifics of deadenylation may show differences. Furthermore, the miRNA landscape of humans is much more diverse with the current estimations at 2603 miRNAs in humans compared to 466 in *Drosophila* (miRBase release 21, 2014). Although it is important to note that recent research mining more than 1000 small RNA-seq samples has suggested >3000 novel miRNAs in the human genome (Londin et al., 2015), demonstrating the potential diversity of miRNA mediated regulation.

Another limitation in RNA decay work specifically is the absence of Dis3L1 from the *Drosophila* genome. Whilst there is little current Dis3L1 literature, direct comparison of the cellular localisation could represent an issue. As discussed previously the absence of Dis3L1 means that an increased amount of Dis3 is observed in the cytoplasm carrying out exosome-mediated decay whilst this activity is replaced by Dis3L1 in human cells. The functional difference between Dis3 and Dis3L1 is that Dis3L1 lacks endonucleolytic activity which could also signal key differences between human and *Drosophila* cytoplasmic decay.

Sidebar title: Is there functional redundancy between cytoplasmic exoribonucleases? RNA decay was long believed to be a passive method for removal of unwanted RNAs from the cell with little specificity; however, it is now clear that RNA decay is both targeted and specific. Although it is clear that redundancy does exist between the decay pathways through work demonstrating that XRN1 and DIS3L2 co-immunoprecipitate in an RNA-dependent manner (Lubas, et al., 2013), and that Xrn1 mutations are synergistically lethal with Dis3 mutants in yeast (Schneider, et al., 2009), there is a growing body of evidence suggesting that specific RNAs are sensitive to specific decay machines. For example, the specific phenotypes that present following loss of individual RNA stability mediators (summarised in Table 2), suggest defined roles within the cell. For instance, Dis3L2 depletion in an *xrn1/pacman* mutant background has been shown to compensate for tissue growth without changes to each pathway affected (Towler, et al., 2016). The increasing indication that exosome subunits may also be involved in targeting specific transcripts also provides evidence towards subtle specificity achieved through regulating complex composition (Boczonadi, et al., 2014; Di Donato, et al., 2016; Eggens, et al., 2014; Kiss & Andrulis, 2010; Wan, et al., 2012).

CONCLUDING REMARKS

Here we have discussed the current body of *Drosophila* work which has aided immensely the understanding of how RNA stability regulation is achieved across eukaryotes. However, we still have much to learn about the intricacies of these regulatory events; for example, many *cis*-acting elements together with the specific RNA binding proteins they recruit remain poorly understood. Understanding these targeting factors together with further work on the coupling between translation and RNA decay will undoubtedly shed more light on how RNA stability is regulated on a specific basis.

Many of the examples discussed in this review have demonstrated the benefits of the rapid expansion of tools available to *Drosophila* researchers to facilitate the investigation of the mechanisms conferring regulation over RNA stability. For example, the reduction in cost of RNA-sequencing now enables many groups to access this method of screening for transcriptional and post-transcriptional changes in gene expression in whole tissues/organisms. Recently SLAM-seq, an exciting tool developed in *Drosophila* S2 cells, enables researches to monitor RNA half-lives *in vivo* (Herzog, Reichholf, & Ameres, 2017). Although its use was shown in S2 cells, as long as tissue 4-thioU uptake is consistent this development could open up an area of research that was notoriously difficult in whole organism studies. In addition to differential gene expression analyses, the ground breaking CRISPR/Cas9 has been developed for use in *Drosophila* (Bassett, et al., 2013). First performed in *Drosophila* by Bassett *et al* there are now a number of tools facilitating genome editing in the whole fly. The use of CRISPR/Cas9 alongside the *GAL4-UAS* system has made *Drosophila* an even more attractive model organism to study the highly conserved mechanisms regulating RNA stability.

ACKNOWLEDGMENTS

The authors would like to thank Amy Pashler, Elisa Bernard, Chris Jones and Jose Pueyo Marques for critical reading of the manuscript. This work was funded by the Biotechnology and Biological Sciences Research Council (BB/P021042/1).

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